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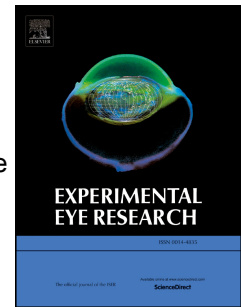
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**Aged complement factor H knockout mice kept in a clean barriered environment
have reduced retinal pathology**

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Abstract

Age-related macular degeneration (AMD) is the largest cause of visual loss in those over 60 years in the West and is a condition increasing in prevalence. Many diseases result from genetic/environmental interactions and 50% of AMD cases have an association with polymorphisms of the complement system including complement factor H. Here we explore interactions between genetic predisposition and environmental conditions in triggering retinal pathology in two groups of aged complement factor H knock out (*Cfh*^{-/-}) mice. Mice were maintained over 9 months in either a conventional open environment or a barriered pathogen free environment. Open environment *Cfh*^{-/-} mice had significant increases in subretinal macrophage numbers, inflammatory and stress responses and reduced photoreceptor numbers over mice kept in a pathogen free environment. Hence, environmental factors can drive retinal disease in these mice when linked to complement deficits impairing immune function. Both groups of mice had similar levels of retinal amyloid beta accumulation. Consequently there is no direct link between this and inflammation in *Cfh*^{-/-} mice.

Keywords; Age-related macular degeneration, Factor H, pathogens, inflammation

1. Introduction

Age-related macular degeneration (AMD) is a condition increasing in prevalence ([Klein, et al., 1997](#), [Klein, et al., 2004](#)) and the major cause of visual loss in those over 60 years in the Western world ([Klein, et al., 2004](#)). In AMD there are major changes in the subretinal area with chronic inflammation and the accumulation of lipid and amyloid beta (A β) rich deposits on Bruch's membrane (BM) under the retinal pigmented epithelium (RPE) ([Johnson, et al., 2002](#)). In association with these deposits there is a reduction in the ability to exchange nutrients and oxygen between the choroidal blood supply and the retina, contributing to central retinal atrophy ([Booi, et al., 2010](#), [Curcio CA, 2013](#), [Guymer, et al., 1999](#)). In a minority of cases the disease can progress with development of new choroidal blood vessels which leak, producing subretinal haemorrhages that may result in profound visual loss ([Rodrigues, 2007](#)).

Polymorphisms of factor H are found in approximately 50% of AMD cases. Factor H is part of the innate immune system. Hence, there may be potential immunological compromise in such individuals ([Edwards, et al., 2005](#), [Hageman, et al., 2005](#), [Klein, et al., 2005](#)). However, it is unclear why some with polymorphisms acquire disease while others do not. Many studies have evaluated risk factors for AMD pathogenesis, ([Cipriani, et al., 2012](#), [Francis and Klein, 2011](#), [Friedman, et al., 2004](#), [Vingerling, et al., 1996](#)), but one hypothesis is that environmental factors could trigger disease in genetically susceptible individuals with compromised immunity producing chronic inflammation. Inflammation is a common risk factor ([Hageman, et al., 2001](#)) and there is evidence of macrophages, lymphocytes and mast cells in the outer retina with disease progression ([Hageman, et al., 2001](#), [Penfold, et al., 1985](#)). While there have been suggestions that environmental factors may play a role in AMD, this critical question remains unresolved

with evidence both for ([Johnson, et al., 2006](#),[Robman, et al., 2010](#),[Seddon, et al., 2004](#),[Seddon, et al., 2005](#)) and against ([Klein, et al., 2008](#),[McGwin, et al., 2005](#),[Schaumberg, et al., 2006](#)).

The complement factor H knock out mouse (*Cfh*^{-/-}) has a distinctive ocular phenotype, showing age-related retinal degenerative changes both in structure and function, and is used as a disease model ([Coffey, et al., 2007](#),[Lundh von Leithner, et al., 2009](#)). It has the key advantage in studying gene-environment interactions because it has greater genetic homogeneity than found in the human population. Here, we ask if environmental factors contribute to retinal degenerative changes in this model. We mature *Cfh*^{-/-} mice from birth to 9 months in either a specific pathogen free environment (SPF), or a conventional open environment (CE). We then examine multiple markers of inflammation and stress and map these against changes in the outer retinal photoreceptor population.

2. Material and Methods

2.1. Ethics Statement

All animals were used with University College London ethics committee approval and under a UK Home Office project licence (PPL 70/8379). All animal procedures were conformed to the United Kingdom Animal License Act (1986) and local regulations

2.2. Animals

Forty 9 months old female homozygous *Cfh* knockout mice on a C57Bl/6J background (*Cfh*^{-/-}) were used. These originated from the same colony which were previously backcrossed for more than 10 generations onto C57Bl/6J animals ([Alexander, et al.,](#)

2005, Coffey, et al., 2007). Mice were a gift originally from Matthew Pickering at Imperial College London. Two breeding groups were separated and put in two different environments; one was retained in a specific pathogen free (SPF) environment (n=20 mice) and the other in a conventional unit (CE) (n=20 mice), exposed to an open environment. Both were maintained on the same diet and housed under the same standard lighting regime. Genotyping of these mice confirmed that the *Cfh*^{-/-} mice from both groups were complement factor H negative on a BL/6J background.

2.3. Immunohistochemistry of flatmounts

Mice were sacrificed by cervical dislocation and eyes were processed for different experiments. For immunostaining of the RPE-choroidal flatmounts (n = 10 eyes per group), enucleated eyes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 1h. The cornea, lens and retina were removed and 5-7 radial cuts made in the RPE-choroidal tissues to produce flat mounts. After several PBS washes, the RPE-choroidal tissues were blocked and permeabilised with 5% Normal Donkey serum in 3% (v/v) Triton X-100 in PBS for 2h. Samples were incubated overnight in a rabbit polyclonal antibody to Iba-1 (1:1000, MP-290-CR01, A. Menarini Diagnostics) made in 1% Normal Donkey Serum in 3% Triton X-100 in 0.1M PBS. After primary antibody incubation, samples were washed repeatedly in PBS and incubated in a secondary antibody, Alexa Fluor donkey anti rabbit 568, made up in 2 % Normal Donkey Serum in 0.3% Triton X-100 in PBS at a dilution of 1:2000, and incubated for 2h. After the secondary antibody incubation, samples were washed repeatedly in PBS and nuclei stained with 4', 6-diamidino-2-phenylindole (Sigma) for 1 min. Tissues were then washed in PBS and repeatedly in Tris buffered Saline (pH 7.5). The flat tissues were then mounted in Vectashield (VECTOR Laboratories) and coverslipped. Tissues were

viewed using Epi-fluorescence and images captured as 24-bit colour images at 3840x3072 pixel resolution using a Nikon DXM1200 digital camera. Iba-1 positive cells were counted across the entire RPE surface at a magnification of X400.

2.4. Immunohistochemistry for stress markers (GFAP and vimentin), inflammatory markers (TNF α and IL-1 β), amyloid beta and cell counts

Eyes (n=5 per group) were fixed in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose and cryosectioned and immunostained as undertaken previously by Hoh Kam et al. (Hoh Kam, et al., 2010) to reveal levels of retinal inflammation with a rabbit polyclonal antibody to interleukin 1 beta (IL-1 β) (1:200, ab9722, Abcam) and a rabbit polyclonal antibody to TNF- α (1:1000, ab1793, Abcam). For the stress markers, retinal sections were immunostained with a mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) (1:200, G3893, Sigma-Aldrich, UK) and a rabbit monoclonal antibody to vimentin (1:200, ab 92547, Abcam). Sections were also immunostained with a mouse monoclonal antibody to amyloid beta 4G8 (1:500, 800701, Covance) which accumulates with age in the outer retina (Hoh Kam, et al., 2010). Sections were visualised using either the fluorescent conjugate Alexa Fluor 568 (1:2000, Invitrogen) or 488 (1:2000, Invitrogen). Additional retinal sections from these animals were also stained with a nuclear marker 4', 6-diamino-2-phenylindole (DAPI) (Sigma), to identify photoreceptor nuclei.

Retinal sections were also stained with peanut agglutinin (PNA) that labels cone membranes around the inner and outer segments. Sections were briefly washed with PBS and incubated with PNA conjugated with Alexa Fluor 488 (1:100, Invitrogen),

made in 0.1M PBS, for 1h followed by several brief washes with PBS. The slides were mounted in Vectashield (Vector Laboratories) and coverslipped.

2.5. Western Blot

Eyes (n=10 per group) were dissected on ice and retinal and RPE-choroidal tissues including the sclera were separated and snap frozen in liquid nitrogen. Samples were homogenised in 2% SDS with protease inhibitor cocktail (Thermo Scientific) using a sonicator and centrifuged at 13,000 X g for 30 mins. The supernatant was transferred to a new microcentrifuge tube and protein concentration measured with an absorbance of 595nm. Bovine serum albumin was used as a standard protein concentration.

Proteins were separated by a 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The nitrocellulose membranes were pre-treated with 5% non-fat dried milk in 1M PBS (pH7.4) for several hours and incubated overnight at 4°C with the following antibodies: rabbit polyclonal antibody to TNF- α (1:3000, ab1793, Abcam), rabbit polyclonal antibody to IL-1 β (1:500, ab9722, Abcam), mouse monoclonal antibody to GFAP (1:500, G3893, Sigma-Aldrich) and rabbit monoclonal antibody to Vimentin (1:500, ab 92547, Abcam) followed by several washes in 0.05% Tween-20 in 1M PBS. The membranes were then incubated with the respective peroxidase conjugated secondary antibodies: Goat anti Rabbit HRP (1:2000, DakoCytomation) and a goat anti mouse HRP (1:10,000, Thermo Scientific) for 1h. Protein immunoreactivities were visualised by exposing x-ray films to blots incubated with ECL reagent (SuperSignal West Dura, Thermo Scientific). Total protein profile was determined by staining blots with Ponceau S solution to check the transfer efficiency. The protein loading control used here was alpha-tubulin (1:2000, T5168, Sigma Aldrich UK). Protein bands were then photographed and scanned.

The absolute intensity of each band was then measured using Adobe Photoshop CS5 extended.

2.6. Data Analysis

2.6.1. Counting of Iba-1 positive cells in the subretinal area

Iba-1 positive macrophages/microglia were counted across the entire outer retinal eye cup en face on the RPE surface.

2.6.2. Measurement of the expression of levels of inflammation, stress markers and A β in the retina by immunostaining

Analysis of immunostaining was focussed on the central retina. Fluorescence images were taken in JPEG format at X400 using an Epi-fluorescence bright-field microscope. Images were montaged and the pixel density was recorded using Adobe Photoshop CS5 extended. The lasso tool was used to draw around areas of interest. All images used in the analysis were derived from central retinal regions. For each marker the intensity of label was measured in retinal layers where the targeted proteins were clearly localised.. Hence, for TNF- α , IL-1 β and amyloid beta this was the outer retina, while for GFAP and vimentin it was the inner retina.

2.6.3. Measurement of the amount of inflammation and stress markers in Western blots

Scanned images of immunoblots were inverted to grayscale format and the mean grey value was measured for each protein band by using the lasso tool to draw a line all the way around the edges of the band using Adobe Photoshop CS5 extended. The absolute intensity was calculated by multiplying the mean grey value and the pixel value. The

protein bands were quantified and their ratios to alpha tubulin (α -tubulin) were calculated and plotted into graphs.

2.6.4. Retinal measurements and photoreceptor outer segment numbers

Multiple counts of the number of photoreceptor nuclei spanning vertically across the outer nuclear layer thickness in the central retina were made. Five adjacent strips were counted in 5 retinae per group on both sides of the optic nerve head. The number of PNA positive inner/outer segments was also counted in central retinal strip of 80 μ m from 5 mice per group.

2.6.5. Statistical analysis

For comparison between the two groups, a Mann-Whitney *U* test was used. Data were analysed using GraphPad Prism version 5.0 for windows (GraphPad, San Diego, USA).

3. Results

3.1. Influence of environmental conditions and genetic background on subretinal macrophages accumulation

The apical side of the RPE of pathogen free (SPF) and conventional environment (CE) mice were immunostained with a macrophage/microglia marker Iba-1 (Hoh Kam, et al., 2010). This is upregulated when macrophages/microglia are activated ([Sasaki, et al., 2001](#)). It is noted that the number of these cells on the RPE surface increases progressively with age in normal C57 mice, when either kept in a pathogen free or conventional environment (data not shown). In age matched C57 mice around 50 macrophages were found. An average of 8 macrophages were identified in the subretinal space of *Cfh*^{-/-}-SPF animals while in *Cfh*^{-/-}-CE mice, the number significantly

increased to approximately 110 in each eye ($P < 0.001$, Fig. 1). Hence, differences between the two environments have a marked impact on outer retinal macrophage number. The rationale for both the relative reduction and increase may be that when the immune response in the *Cfh*^{-/-} mice is challenged by an open environment, the number of macrophages increases in *Cfh*^{-/-}-CE mice but in the total absence of any challenge in the SPF environment, the *Cfh*^{-/-} mice fail to activate an immune response in terms of these cells. This is also the probable reason for the difference in the numbers of macrophages between the *Cfh*^{-/-} in both environments, and C57 animals.

3.2. Influence of environmental conditions and genetic background on inflammation

The above results revealed significantly elevated subretinal macrophage numbers in the conventional environment in *Cfh*^{-/-} mice, implying that there might be a retinal inflammatory response not present in those barriered from the environment. Tumour necrosis factor alpha (TNF- α) is an important pro-inflammatory cytokine produced mainly in monocytes and activated macrophages (Brouckaert, et al., 1993). It can stimulate RPE cells to produce monocyte chemoattractant protein (MCP-1) to recruit macrophages to the sub-RPE space (Bian, et al., 1999; Elner, et al., 1991). Therefore we immunostained retinas with TNF- α to determine if relative levels in the two groups of mice reflected the Iba-1 data (Fig. 2 A-B). TNF- α was expressed predominantly in the photoreceptor layers of both groups of mice, consistent with the subretinal accumulation of macrophages. However, consistent with our macrophage data, *Cfh*^{-/-}-CE mice had significantly higher TNF- α levels in the outer retina than their fellow mice kept in the barriered environment ($P < 0.0001$) (Fig. 2A-B). Western blot results revealed a

protein band size of 26kDa which relates to transmembrane TNF- α , a precursor of the soluble form of TNF- α . Transmembrane TNF- α is expressed on activated macrophages and lymphocytes (Perez, et al., 1990) and plays a critical role in local inflammation (Arora, et al., 2009, Kaymakcalan, et al., 2009, Mitoma, et al., 2004, Mitoma, et al., 2008). Western blot results mirrored those found with immunostaining showing that *Cfh*^{-/-}-CE mice have an increased level of transmembrane TNF- α compared to *Cfh*^{-/-}-SPF animals. However, statistical significance here was not achieved because of higher levels of variability in the Western blot data derived from the whole eye cup compared to the immunostaining ($P>0.05$, Fig. 2C).

We also examined staining for another pro-inflammatory cytokine, IL-1 β (Fig. 2D-E), which is synthesized in activated macrophages and is involved in the inflammatory response (March, et al., 1985, Mochizuki, et al., 1987, Oppenheim, et al., 1986). IL-1 β is expressed in the ganglion cell layer, inner nuclear layer and the outer retina. In *Cfh*^{-/-}-CE mice, the fluorescent signals for IL-1 β increased in all layers compare to *Cfh*^{-/-}-SPF animals. When the expression of IL-1 β was measured in the outer retina, significantly more was expressed in *Cfh*^{-/-}-CE mice ($P<0.05$). Western blot from whole eye cups confirmed the relative difference between the groups but this was not statistically significant. However, Western blot results also show that both the inactive pro IL-1 β (31kDa) and the active mature (17kDa) IL-1 β were present in the retina of both groups of mice, which could not be separated, but also may have contributed to the lack of statistical significance as we measure both protein bands as total IL-1 β .

Influence of environmental conditions and genetic background on stress markers

Next we assessed qualitatively and quantitatively the level of stress markers, GFAP and vimentin, in the two groups of mice by both immunostaining and Western blots. GFAP

and vimentin are cytoskeletal intermediate filaments expressed in retinal Müller cells and are upregulated following stress and ageing (Fisher, et al., 2005, Hippert, et al., 2015). GFAP positive intermediate filaments seen in the immunostaining were largely restricted to astrocytes at the inner retinal margin, adjacent to the ganglion cell layer (GCL) where they were measured (Fig. 3A). There was a significant increase in GFAP in the animals kept in the conventional environment compared to those kept in the specific pathogen free environment ($P < 0.01$, Fig. 3B). Western blot analysis from whole eye cups revealed the same trend as that obtained from the immunostaining but this was not statistically significant ($P > 0.1$, Fig. 3C). When retinal sections from both groups were immunostained with vimentin, positive Müller glial fibres could be seen extending up into the inner nuclear layer (INL) as far as the outer plexiform layer (OPL) in both groups (Fig. 3D). It was obvious that there were marked differences between the groups that could be measured in multiple ways. Analysis of vimentin staining revealed a significantly higher level in retinae of the *Cfh*^{-/-}-CE mice compared with *Cfh*^{-/-}-SPF animals ($P < 0.05$, Fig. 3E). Western blot analysis from the whole eye cups showed the same trend in the *Cfh*^{-/-}-CE mice although this was not statistically significant ($P > 0.1$, Fig. 3F).

3.3. The effect of environmental conditions and genetic background on photoreceptors number

If there is an inflammatory response and an increase in stress level specifically in the retinae of *Cfh*^{-/-}-CE animals, do these result in elevated degenerative changes in the outer retina? Counts of photoreceptor nuclei at equatorial locations in the retina were undertaken vertically across the outer nuclear layer (ONL) as these are vulnerable to outer retinal inflammation and undergo apoptosis early in AMD (Dunaief, et al., 2002).

Differences were found between the two groups of mice. The *Cfh*^{-/-}-CE animals in which there was elevated inflammation and stress levels had significantly reduced number of ONL cells ($P < 0.01$, Fig. 4A-B) compared to the *Cfh*^{-/-}-SPF mice. Retinae were also stained with peanut agglutinin (PNA), a cone photoreceptor marker ([Blanks and Johnson, 1983](#)). Cones form only 1-3% of the mouse photoreceptor population ([Carter-Dawson and LaVail, 1979a](#), [Carter-Dawson and LaVail, 1979b](#)) but are more vulnerable to inflammation and stress than rods ([Francis, et al., 2005](#), [Holder, et al., 2005](#), [Mantel, et al., 2008](#), [Scholl and Zrenner, 2000](#)). Cone numbers in *Cfh*^{-/-}-CE mice where inflammation and stress levels were elevated were significantly reduced compared with *Cfh*^{-/-}-SPF animals ($P < 0.01$, Fig. 4C-D).

Although cone numbers are significantly reduced in the *Cfh*^{-/-}-CE mice compared to SPF animals, because they form such a small proportion of the total photoreceptor population, the significant reduction in nuclei number samples in the ONL strongly implies that there is also rod photoreceptor loss.

3.4. The effect of environmental conditions and genetic background on amyloid beta deposition on Bruch's membrane

Mice deposit amyloid beta ($A\beta$) along the RPE/BM with age and this is substantially greater at all ages in *Cfh*^{-/-} than normal C57 animals ([Catchpole, et al., 2013](#)). In *Cfh*^{-/-} mice it is present from 6 months ([Catchpole, et al., 2013](#)). In spite of the consistent and significant differences between the *Cfh*^{-/-}-CE and the *Cfh*^{-/-}-SPF mice revealed above, both groups displayed the same age-related $A\beta$ accumulation along the RPE/BM interface and on their photoreceptor outer segments (Fig. 5). Hence, this reveals a dissociation between the development of pathology and $A\beta$ load. Consequently $A\beta$ is unlikely to be the triggering factor for retinal inflammation alone in the *Cfh*^{-/-}-CE mice.

4. Discussion

Our results demonstrate age related retinal differences in a genetically compromised mouse maintained in either a standard open environment compared to the same animals kept in a closed pathogen free environment. Those kept in a conventional environment showed significantly greater number of macrophages, higher levels of inflammation and retinal stress and reduced photoreceptor numbers. These differences were independent of A β load which was the same in both groups. This suggests that environmental factors may play a role in retinal degenerative changes in this mouse model. These differences reported here were not identified in wild type C57Bl/6 mice kept in similar environments, presumably because they were not immune compromised (data not presented).

The *Cfh*^{-/-}-CE mice have a significantly greater number of subretinal macrophages and expressed higher levels of inflammatory markers, TNF- α and IL-1 β than found in *Cfh*^{-/-} - SPF mice. This is consistent with these animals having a chronic pro-inflammatory condition. They also have elevated GFAP and vimentin associated with increased retinal stress. Monocytes and activated macrophages are the main cells that produced TNF- α , a pro-inflammatory cytokine, (Brouckaert, et al., 1993). This causes RPE cells to synthesise MCP-1 increasing macrophages recruitment ([Bian, et al., 1999](#), [Elner, et al., 1991](#)). Similarly, in the brain microglia and astrocytes activated by pathogens express and release high levels of TNF- α ([Jekabsone, et al., 2006](#), [Lotz, et al., 2005](#), [Medeiros and LaFerla, 2013](#), [Walter, et al., 2007](#)) stimulating microglial proliferation ([Mander, et al., 2006](#)). This may explain the relatively high level of TNF- α and high number of subretinal macrophages in the *Cfh*^{-/-}-CE mice. The precursor form of TNF- α , that we identify in our Western blots is transmembrane TNF- α , which is also involved in the

inflammatory response. It exerts its influence via direct cell to cell contact, binding to the TNF receptors on the target cells (Perez, et al., 1990), and can influence microglial phagocytic capacity (Neniskyte, et al., 2014). Studies of microglia in prion disease brains have some relevance to our findings. These are not necessarily pro-inflammatory. However, when challenged by systemic inflammation they switch from a primed to an aggressive pro-inflammatory phenotype associated with neuronal apoptosis ([Teeling and Perry, 2009](#)).

IL-1 β is a potent pro-inflammatory cytokine that initiates and amplifies a wide variety of effects associated with innate immunity and host responses to microbial invasion and tissue injury (Dinarello, 1996). It is expressed in both the inner retina and in relation to photoreceptors of both the open environment and barriered mice. This is different from the expression pattern of TNF- α which is mainly present in association with the photoreceptor population. IL-1 β production and release are stimulated by either pathogen-associated molecular pattern molecules or damage-associated molecular pattern molecules and involve an initial signal to induce up-regulation of the inactive precursor pro- IL-1 β (31kDa) and a second signal to cleave the pro-IL-1 β into its mature form IL-1 β (17kDa) which is then secreted (Schroder and Tschopp, 2010).

GFAP and vimentin that were upregulated in mice kept in an open environment form intermediate filaments as part of the cytoskeleton in astrocytes and Müller cells. The production of these increases in reactive astrocytes and Müller cells under stress ([Barker, et al., 1996](#), [McGraw, et al., 2001](#)) contributing to retinal degeneration ([Kim, 2015](#)).

Aged photoreceptors experience a level of high metabolic stress and progressive inflammation. ([Graymore, 1969](#), [Lundh von Leithner, et al., 2009](#)). It is also one in

which, there is progressive cell death with 30% photoreceptor cell loss in rodents (Cunea and Jeffery, 2007, Cunea, et al., 2014). This cell loss is most likely related to progressive inflammation. Our *Cfh*^{-/-}-CE had higher levels of retinal stress and inflammation and this was associated with a reduction in photoreceptors compared with the *Cfh*^{-/-}-SPF animals. This loss likely includes both rods and cones. Rods form about 97% of the mouse photoreceptor population (Carter-Dawson and LaVail, 1979a, Carter-Dawson and LaVail, 1979b) and it is unlikely that the significant reduction in photoreceptors could take place without impacting on them. However, our data also show specific cone loss when cells are PNA labelled, which is a cone specific marker. The loss of cones may be expected as there is evidence that retinal inflammation impacts more significantly on them than rods (Francis, et al., 2005, Holder, et al., 2005, Mantel, et al., 2008, Scholl and Zrenner, 2000).

Recently, significant evidence has accumulated showing that Alzheimer's disease (AD) and neuronal degeneration can be driven by systemic infection (Holmes and Cotterell, 2009, Perry, et al., 2010). However in AD this is not restricted to a specific agent, rather there is evidence for substantial associations with bacterial ([Maheshwari and Eslick 2015](#); Miklossy and McGeer, 2016), fungal ([Pisa, et al., 2015](#)) and viral (Itzhaki, et al., 2016; [Harris and Harris, 2015](#); [Steel and Eslick, 2015](#)) agents. These include oral infections that increase levels of circulating cytokines (Cestari, et al., 2016; Ide, et al., 2016). Hence, a wide range of pathogens can drive AD and they can be focused in distal tissues. We propose that similar events may occur in our immune compromised mice, and possibly also in some cases of AMD. In normal mice, inflammation is likely to lead to adaptive homeostatic immune and metabolic changes. However, in those that are immune compromised homeostatic mechanisms are undermined and systemic

inflammation has an exaggerated response leading to cell death ([Teeling and Perry, 2009](#)). Hence, searching for specific agents is unlikely to be productive. It is likely that, as with AD, infection induced by a variety of agents will impact on the retinae of our mice and it may be the total pathogen load that is significant, not the influence of one specific pathogen.

From data not presented, we know there are no differences in the metrics examined here in C57 mice kept in either environment. However *Cfh*^{-/-} animals have compromised immune systems and likely suffer more when environmentally challenged. The rate of age related photoreceptor loss in these mice is likely modulated by progressive inflammation that may be driven by many diverse elements in an open environment.

This study has shown that in the *Cfh*^{-/-} mouse model, disease progression can be driven by the environmental factors. However, there was no difference in A β load between the two groups of mice. Hence, A β is not a primary factor in disease progression in this mouse model. This is important because retinal A β removal has become a key therapeutic strategy via immunotherapy ([Catchpole, et al., 2013](#), [Ding, et al., 2011](#), [Ding, et al., 2008](#)). The rationale for this is based on the neurotoxic aspect of A β ([Chiang, et al., 2008](#), [Lesne, et al., 2006](#)). However, lighter A β oligomers are functional and modulate synaptic plasticity. Their removal is linked to cognitive decline ([Parihar and Brewer, 2010](#); [Puzzo, et al., 2012](#)). Synaptic plasticity is a feature of photoreceptor ribbon synapses ([Haverkamp, et al., 2006](#); [Aartsen, et al., 2006](#); [Sanyal, et al., 1992](#); [Jansen and Sanyal, 1992](#)) and A β removal is associated with reduced inner retinal responses ([Ho, et al., 2012](#)). Hence, elements of A β are likely to be functional and independent of inflammation and retinal pathology

But it is likely that a complex relationship exists between environmentally driven inflammation and A β deposition that has yet to be revealed.

Disclosure statement

The authors declare no conflict of interest. All animals were used with University College London ethical committee approval and under a Home Office animal project license. All animal procedures conformed to the United Kingdom Animals Scientific Procedures Act 1986.

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Figure Legends

Figure 1. *Cfh*^{-/-} mouse exposure to a conventional environment (CE) increases the number of Iba-1 positive cells in the subretinal region compared with mice maintained in a specific pathogen free environment (SPF). Retinal flatmounts were immunostained with an antibody to Iba-1 marking macrophages (red). The number of Iba-1 positive cells in the two groups of *Cfh*^{-/-} mice. *Cfh*^{-/-} -CE mice had significantly elevated number of macrophages (mean ~110) in comparison with the *Cfh*^{-/-} -SPF animals (mean ~10) ($P < 0.001$). There were no differences in the morphology or the distribution of the cells labelled in the two groups. The lower right hand panel shows a representative macrophage at higher power. Graphs show Mean \pm SEM. Scale bar = 50 μ m for upper panels and 20 μ m for the lower panel.

Figure 2. *Cfh*^{-/-} mouse exposure to a conventional environment (CE) results in an increase in inflammation compared with mice maintained in a specific pathogen free environment (SPF). **A.** Retinal sections from the two groups of *Cfh*^{-/-} mice were immunostained for TNF- α , a pro-inflammatory cytokine (Red) and the nuclei were counterstained with 4', 6-diamino-2-phenylindole (DAPI) (Blue). **B.** Graphs showing levels of TNF- α in retinae of the two groups of mice. There was a significantly high level of TNF- α in the photoreceptor layer of the *Cfh*^{-/-}-CE mice compared to *Cfh*^{-/-}-SPF animals ($P < 0.0001$). **C.** Quantification of TNF- α protein by Western blot for the whole eye cup including sclera and choroid. This confirmed that CE mice had higher TNF- α protein levels but this did not reach statistical significance perhaps because analysis was for the whole eye cup diluting the targeted protein ($P > 0.05$) **D.** Retinal sections for the same groups immunostained with IL-1 β (green) and the nuclei counterstained with DAPI (blue). IL-1 β was present in the GCL, the IPL and the PR layers in both groups but the expression was stronger in *Cfh*^{-/-}-CE-retinae. **E.** Quantification of IL-1 β measuring the

pixel intensity with immunostaining in the outer retina. This shows a significant increasing in IL-1 β in the *Cfh*^{-/-}-CE mice ($P < 0.05$). **F.** Western blot results for IL-1 β confirmed the trend found with immunostaining but was not statistically significant ($P > 0.1$). Mean \pm SEM. Scale bar = 25 μ m. GCL; ganglion cell layer, INL; inner nuclear layer, ONL; outer nuclear layer, PR; photoreceptor layer.

Figure 3. *Cfh*^{-/-} mouse exposure to a conventional environment (CE) results in an increase in retinal stress compared with mice maintained in a specific pathogen free environment (SPF). Retinal sections from the two groups of *Cfh*^{-/-} mice were immunostained for GFAP and vimentin stress markers. **A.** Representative micrographs of retinal sections immunostained with GFAP (Red) and the nuclei counterstained with DAPI (blue). The GFAP positive intermediate filaments within the retina were largely restricted to astrocytes at the inner retinal margin, adjacent to the ganglion cell layer (GCL) in both groups of mice. **B.** Measurement of GFAP levels in the two groups of mice. There was a significant increased expression of GFAP in the *Cfh*^{-/-}-CE mice compared to the *Cfh*^{-/-}-SPF animals ($P < 0.01$). **C.** Western blot analysis for protein revealed a similar increase although this was not statistically significant probably because whole eye cup preparations were used including the choroid and sclera diluting the targeted protein. **D.** Retinal sections immunostained with vimentin (green). Vimentin positive Müller glial fibres could be seen extending up into the inner nuclear layer (INL) as far as the outer plexiform layer (OPL) in both groups of mice. **E.** Measurements of vimentin levels in the retina of both groups of mice, revealing a statistically significant difference between groups with greater levels in the retina of the *Cfh*^{-/-}-CE mice compared to *Cfh*^{-/-}-SPF animals ($P < 0.05$). **F.** Western blot analysis revealed a similar trend to immunostaining but this was not statistically significant. ($P > 0.1$). Mean \pm SEM. Scale bar = 25 μ m. GCL;

ganglion cell layer, IPL; Inner plexiform layer, INL; inner nuclear layer and OPL; outer plexiform layer.

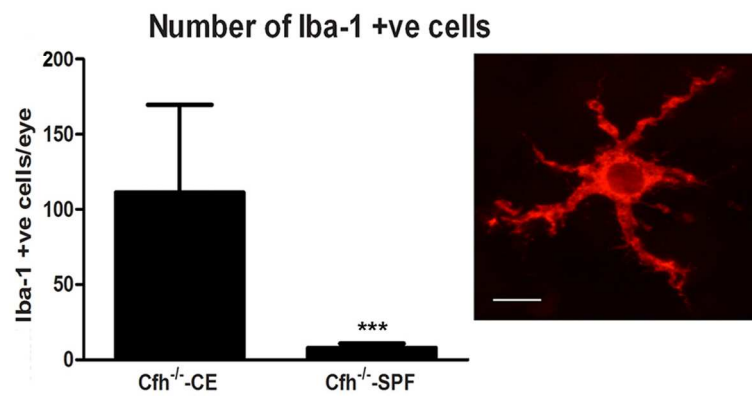
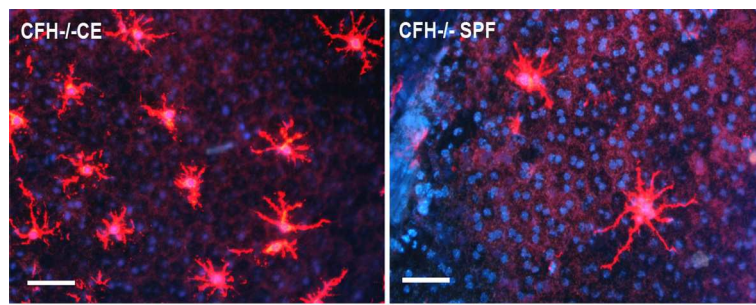
Figure 4. *Cfh*^{-/-} mouse exposure to a conventional environment (CE) have fewer photoreceptors compared with mice maintained in a specific pathogen free environment (SPF). Retinal nuclei were stained with 4', 6-diamino-2-phenylindole (DAPI) (**A.**) and their number counted vertically across the ONL thickness at the same central locations in the two groups of mice. **B.** Graph showing the number of photoreceptor nuclei counted in strips vertically across the ONL thickness in the two groups. There was a significantly relative decrease in photoreceptor nuclei in retinae of the CE mice compared to SPF mice ($P < 0.01$). **C.** Cone photoreceptors from the same locations were stained with peanut agglutinin (Green). **D.** Graph showing the number of cones counted per 80µm in both mouse groups. There were significantly fewer cones in the *Cfh*^{-/-}-CE mice compared with *Cfh*^{-/-}-SPF mice ($P < 0.01$). Hence, increased macrophage number, high levels of inflammation and stress in association with the photoreceptor population in *Cfh*^{-/-}-mice maintained in the conventional environment are associated with photoreceptor loss. Error bars represent SEM. Scale bar = 25µm. INL; Inner nuclear layer, ONL; outer nuclear layer, PR; photoreceptor layer.

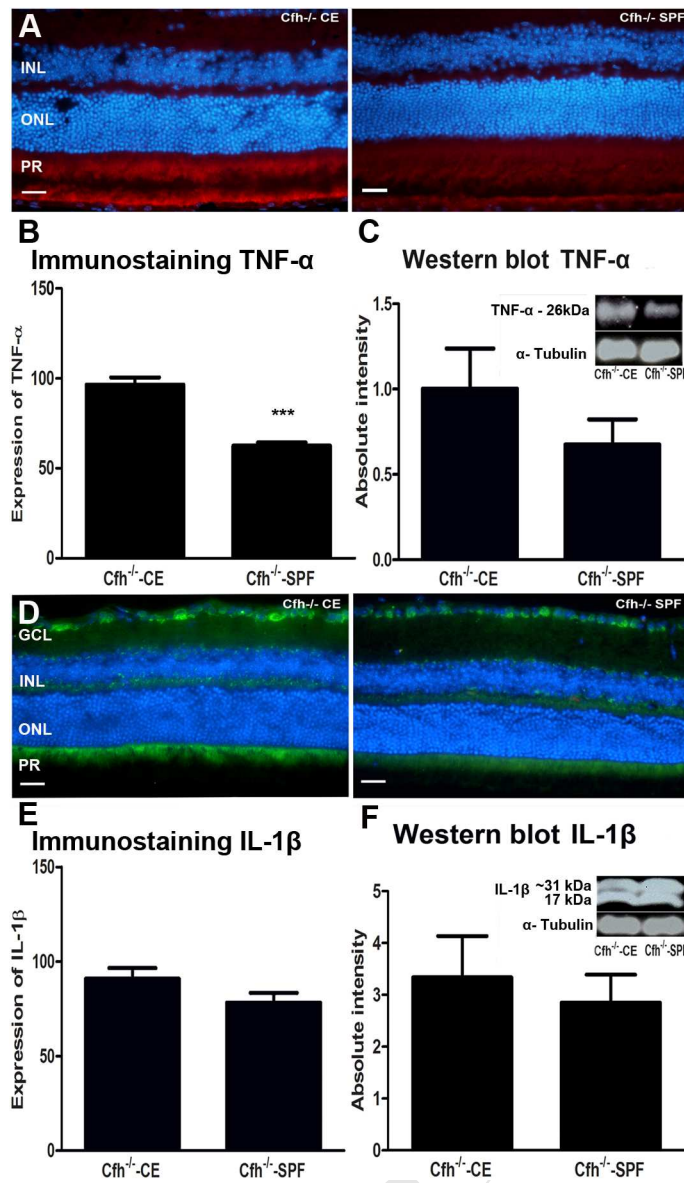
Figure 5. *Cfh*^{-/-} mice maintained in either a conventional environment (CE) or a specific pathogen free environment (SPF) have similar outer retinal amyloid beta levels. A key feature of outer retinal ageing is accumulation of amyloid beta ($A\beta$) along the RPE/BM interface this is a component of drusen that are hallmarks of AMD. There were no significant differences in the accumulation of $A\beta$ along the RPE/BM interface between the two groups of *Cfh*^{-/-} mice ($P = 0.5$). Hence inflammation, stress and cell loss

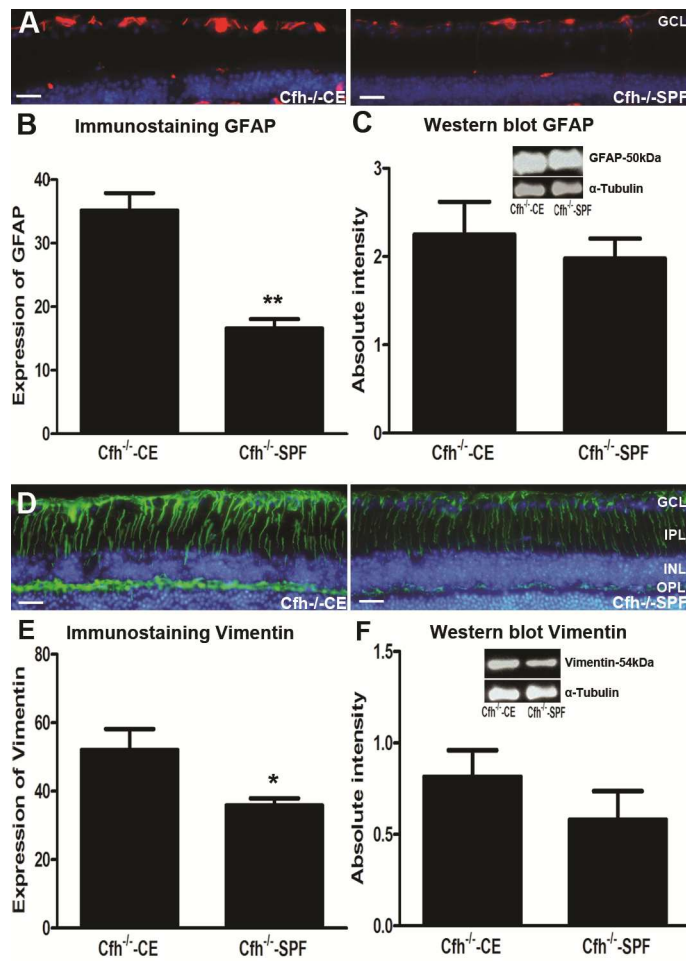
are independent of A β deposition. Error bars represent SEM. Scale bar = 25 μ m. INL;

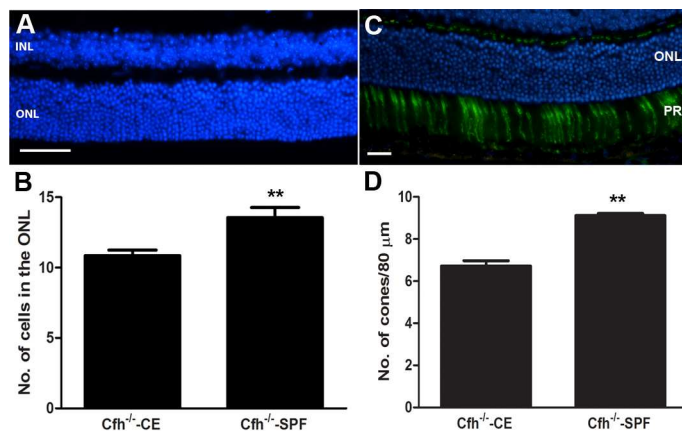
Inner nuclear layer, ONL; outer nuclear layer, PR; photoreceptor layer.

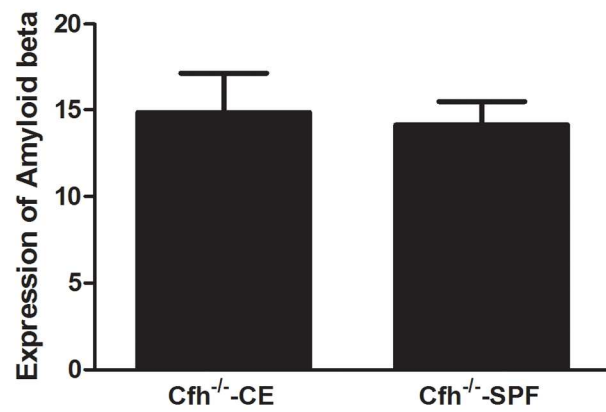
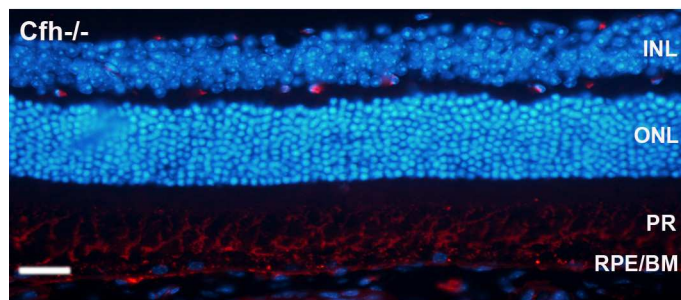
ACCEPTED MANUSCRIPT











- Open environment *Cfh*^{-/-} mice have higher retinal pathology than those barriered
- *Cfh*^{-/-} mice kept in an open environment have fewer photoreceptors when aged
- A β is not an inflammatory driver in *Cfh*^{-/-} as both groups have similar A β levels